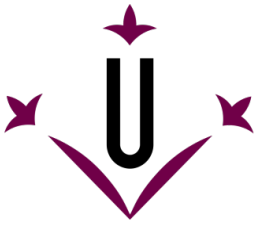


**Short Communication**

# Cytokine gene expression in aborting and non-aborting dams and in their foetuses after experimental infection with *Neospora caninum* at 110 days of gestation

Almeria S[1,2], B. Serrano-Pérez[3], L. Darwich[1,2], R. Mur-Novales[3], I. Garcia-Ispierto[3], O. Cabezón[4], F. López Gatius[3]

[1]Centre de Recerca en Sanitat Animal (CReSA) - Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Campus UAB, 08193 Bellaterra, Barcelona, Spain

[2]Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra, Barcelona, Spain

[3]Departament de Ciencia Animal, Universitat de Lleida. Agrotecnio Center, Lleida, Spain

[4]SEFaS, Dept. Medicina i Cirurgia Animals, Universitat Autònoma de Barcelona (UAB), Spain

-*Corresponding author: Tel: 34 93581 2847, email address: Sonia.Almeria@uab.cat

**Abstract**

*Neospora caninum* is a major cause of abortion in cattle. However, it is not known why not all infected animals abort. In this study, Th1 (IFN-γ), Th2 (IL4) and T reg (IL-10) cytokine gene expression was examined by real time PCR using the TaqMan approach in all of these dams and their foetuses after experimental infection with the isolate Nc-Spain7 at 110 days of pregnancy and euthanasia 6 weeks after infection. In prior published work, foetal death was observed in three of six infected dams and transplacental infection in all the 6 infected foetuses. In the spleen of the dams, IL-4 expression was down-regulated in dams with aborted/non viable foetuses compared to both uninfected dams (controls, n=3) and infected dams with live fetuses at euthanasia. In the lymph nodes draining the placenta, up-regulated expression of IL-4 was observed in infected dams with live foetuses compared to control dams. In the placenta, infected dams with live foetuses had significantly up-regulated IFN-γ in both caruncle and cotyledon and up-regulated IL-10 in cotyledon compared to control dams. Infected live foetuses showed up-regulated expression of IFN-γ and IL-10 in foetal spleen, and showed downregulated expression of IL-4 in the thymus compared to control uninfected foetuses. Expression of any cytokine in the thymus was significantly lower compared to the levels observed in foetal spleen. The results indicate an up-regulated expression of Th1, Th2 and Treg in infected dams with live foetuses and in their foetuses. On the other hand, down-regulation of Th2 immune responses and Treg cytokines were observed in infected dams which had aborted or had non-viable foetuses at euthanasia, suggesting an immunological recovery of cytokine gene expression levels in dams a few weeks after an abortion occurred.

**Keywords: Bovine neosporosis; second trimester of gestation; pregnant cattle; real time RT-PCR; abortion.**

*Neospora caninum* is an obligate intracellular parasite considered a very important cause of abortion in cattle worldwide (Almería and López-Gatius 2013). The precise causes of foetal or placental damage are not well-known and the reasons why some animals abort and other do not remain unclear (Dubey et al., 2006).

Th1 cytokines such as IFN-γ inhibit the multiplication of *N. caninum* tachyzoites inside the cells and has been linked to protection against *N. caninum*-associated abortion in cattle (Almeria et al., 2012). However, pro-inflammatory responses, effective against *N. caninum*, when excessive will likely result in foetal or placental damage (Almeria et al., 2010).

In prior work, we standardized the infection model as an intravenous dose of $10^7$ *N. caninum* tachyzoites for pregnant cows at day 110 of gestation using two different strains (Nc-Illinois in Almeria et al., 2010 and Nc-Spain-7 in Almería et al., 2016). Animals were euthanized 6 weeks after infection. Foetal death was observed in some dams in both experiments (Almeria et al., 2010; Almería et al., 2016), as occurs in field conditions. A protective immune response against abortion could not definitively be associated with IFN-γ levels alone, but significantly lower IFN-γ/IL-4 ratios were observed in the dams with live foetuses (Darwich et al., 2016), highlighting the importance of the Th1/Th2 balance in the course of *N. caninum* protection against abortion in cattle. In our experience, IFN-γ may be detected with a greater sensitivity when the cytokine is determined at the gene expression level (Almeria et al., 2012). The present study analyzed Th1 (IFN-γ), Th2 (IL4) and T reg (IL-10) cytokine gene expression in immune tissues in infected dams with aborted-non viable foetuses and in dams with live foetuses at euthanasia compared to uninfected dams (controls) to try to establish the immune responses taking place in relation to protection against *N. caninum*

abortion. Cytokine gene expression was also determined in foetal tissues and at the materno-foetal interface (caruncle and cotyledon) in infected dams and their live foetuses and control uninfected dams and their live foetuses.

The animals used and the infection protocol have been described elsewhere (Almeria et al., 2016). Briefly, nine 14-16 month-old Holstein-Friesian heifers seronegative for *N. caninum* (CIVTEST, Girona, Spain) were artificially inseminated. Pregnancy was confirmed by ultrasonography 30, 45, 90 and 110 days after insemination. On Day 110 of gestation, six of the heifers were IV inoculated with $10^7$ culture-derived tachyzoites of the *N. caninum* isolate Nc-Spain7. These six animals were euthanized around Day 152 of gestation. The three remaining heifers were kept as un-inoculated controls and were euthanized at the same time as the inoculated dams. Around Day 152 of gestation (Day 42 after infection) all animals were necropsied. Portions of foetal tissues were aseptically obtained and DNA was extracted.

The procedures used in the present study were approved by the Ethics Committee on Animal Experimentation of the Universitat Autonoma de Barcelona (UAB) (license number CEEAH.1426-08/02/2012) and of the Universitat de Lleida (license number CEEA.06-01/12). Animals were handled in accordance with good animal practices and the strict conditions defined by the Animal Ethics Committee at UAB and CReSA, Spain. Every effort was made to minimize suffering.

The lymphatic vessels draining the uterus, the internal iliac lymph nodes, named here as uterine lymph nodes (UTLN) and the spleen were collected from the heifers and spleen and thymus were collected from the foetuses for isolation of mononuclear cells as described by Almeria et al. (2014). In addition, three placentomes –the cranial, medial

and caudal placenta– were recovered from each animal. Both the maternal side of the placenta (caruncle) and the foetal side of the placenta (cotyledon) were careful separated manually from each placentome. Tissues were frozen and homogenized in liquid nitrogen and kept in Trizol at -80º C. Craneal, medial and caudal placental samples collected from each animal were combined and used for RNA extraction to determine cytokine genes in maternal (caruncle) and foetal placenta (cotyledon).

Total RNA was extracted with phenol-cloroform. Samples were treated with DNAse in the presence of RNAse inhibitors for elimination of contaminating genomic DNA and RNA concentrations were determined and RNA integrity was checked. Complementary DNA was synthesized from 2 µg of total RNA and random primers with the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's recommendations.

Messenger RNA expression was determined by real time RT-PCR following the Taqman approach in an ABI PRISM TM 7700 sequence detector (PE Applied Biosystem, Foster city, CA, USA). Probes and primers for bIL4 and bIFN-γ and bIL-10 have been described in Almeria et al. (2003). Primers were designed to span an intron to avoid genomic contamination. Probes and primer pairs were used to quantify GAPDH RNA as the endogenous housekeeping control gene described by Leutenegger et al. (2000).

Probe and primer concentrations for the analyzed cytokines were determined and PCR amplifications were performed as in Almeria et al. (2014). Endogenous GAPDH housekeeping expression was used to normalize levels of cytokine gene expression (Almeria et al., 2012). For relative quantitation of gene expression, the comparative threshold cycle (CT) method (ABI PRISM7700 sequence detection system, user bulletin #2) was used as described in Almeria et al. (2003).

Caruncle and cotyledon samples from the same dams, and thymus and foetal spleen samples from the same foetuses were analysed in the same run to be able to compare their expression levels.

Comparisons between groups (control, infected with live foetuses and infected with aborted/dead foetuses) were tested by a one-way ANOVA test. When statistically significant differences were found Bonferroni´s test was applied to examine all possible pairwise comparisons. Comparisons between two groups (infected with live foetuses versus control uninfected; comparison of caruncle and cotyledon samples; foetal spleen and thymus samples) were performed by Student's t-test. All analyses were done using the SPSS computer package, version 17.0 (SPSS Inc., Chicago, IL). Differences were significant when $p \leq 0{\cdot}05$.

The present study was designed on the basis of the results described in Almeria et al. (2016). In this previous study, of six heifers infected as described here three suffered foetal mortality. All experimentally infected heifers were seropositive to *N. caninum* at euthanasia, and transplacental infection have already taken place in their foetuses. Control uninfected foetuses did not show antibodies and *N. caninum* DNA was not detected in any of their tissues (Almeria et al., 2016).

In the spleen, lower IL-4 mRNA expression was observed between infected dams with aborted foetuses versus infected dams with live foetuses ($p=0.019$) and uninfected dams (controls) ($p=0.025$, one-way ANOVA, Bonferroni-multiple comparison test) (Fig. 1A). The expression of IL-4 was on average 6-fold lower (range 3.4-9.4-folds) in infected dams with aborted foetuses compared to infected dams with live foetuses and an average 2.9-fold lower (range 1.3-6.7-folds) in dams with aborted foetuses versus

uninfected dams (controls). No significant differences were observed for IFN-γ and IL-10 (p=0.09 and p=0.72, respectively, one-way ANOVA, Bonferroni test) (Fig. 1A).

In UTLN, significant differences among groups were observed for IL-4 (p=0.045; one-way ANOVA, Bonferroni test) (Fig. 1B). Compared to control uninfected dams, IL-4 expression was significantly up-regulated in infected dams with live foetuses by 7.3-fold (range 4.7-14.3-fold) (p=0.05, one-way ANOVA, Bonferroni test). No significant differences were observed for IFN-γ and IL-10 (p=0.10 and 0.09, respectively; one-way ANOVA, Bonferroni test) (Figure 1B).

In the cotyledon (foetal placenta), expression levels of IFN-γ and IL-4 were undetectable in control uninfected heifers and low gene expression levels were also observed for IL-10. When normalized levels were compared, significantly increased expression of IFN-γ (p= 0.01; Student's t-test) was observed in cotyledon from infected dams with live foetuses versus uninfected controls. IFN-γ mRNA levels were upregulated 71.2-fold in the foetal placenta from infected dams with live foetuses compared to cotyledon from uninfected dams (range 30-120-fold) (Figure 2). IL-10 mRNA was significantly up-regulated in infected animals by 39.2-fold in the infected group compared to the control uninfected group (range 13.4-136.5) (p=0.028, Student's t-test). Very low expression levels for IL-4 were observed in both groups (average 3.4-fold up-regulation in infected dams with live foetuses; range 1.4-6.9 fold) and no significant differences were observed between groups (p=0.25, Student's t-test). Significant differences between groups were observed in IFN-γ/IL4 ratios on the foetal placenta with the lowest ratios observed in infected foetuses (p=0.005, Student's t-test).

In the caruncle, significantly up-regulated IFN-γ expression (p= 0.03) was observed in infected dams with live foetuses when compared to control uninfected heifers (Fig. 2

and 3). The expression of IFN-γ was on average 25.2-fold higher in infected dams with live foetuses compared to uninfected control foetuses (range 9-51.5-folds). Not significant differences were observed for IL-4 (p=0.09) and IL-10 gene expression between groups (Fig. 3). Significant differences between groups were also observed in IFN-γ/IL4 ratios on the maternal placenta with lowest ratios observed in infected foetuses, (p=0.041, Student's t-test).

The caruncle from the dam with a dead foetus at euthanasia followed the same pattern (higher levels of expression compared to control dams) and had similar levels of cytokines compared to the other dams with infected live foetuses. This dam showed the highest expression of IL-4 in the caruncle of all the analyzed dams (Fig. 3).

No significant differences between caruncle and cotyledon in expression levels were observed in any of the analysed cytokines.

Cytokine expression was evaluated in the spleen and the thymus of control uninfected foetuses and infected foetuses live at euthanasia.

In the foetal spleen, a significant higher IFN-γ mRNA expression (p= 0.02, Student's t-test) was observed in infected foetuses compared to control uninfected foetuses (Figure 4). The expression of IFN-γ was an average 50.7-fold higher in infected live foetuses (range 12.7-385 fold). A trend towards statistical significance was observed for IL-10 (p= 0.06, Student's t-test) with the expression of IL-10 being 7.1-fold higher in the infected group (range 3.2-37 fold). Expression of IL-4 did not show significant differences between groups in the spleen (p= 0.375, Student's t-test). Significant differences between groups were observed in IFN-γ/IL4 ratios on the foetal spleen (p=0.012, Student's t-test).

In the thymus, infected live foetuses showed significantly lower expression of IL-4 than control foetuses (p=0.05, Student's t-test) (Fig. 4). IL-4 expression was 15.4-fold lower in the infected group compared to the control uninfected group (range 7.8-124.5 fold). IFN-γ and IL-10 did not show significant differences in expression between groups (p= 0.74 and p= 0.92, respectively, Student's t-test). High individual variations were observed among animals within each group. Not significant differences were observed in the IFN-γ/IL4 ratios on thymus (p=0.78, Student's t-test).

Based on mean normalized CT values, foetal spleen showed significantly higher expression of IFN-γ and IL-10 (p= 0.025 and p= 0.005, respectively, Student's t-test) and a trend towards statistically higher expression of IL-4 (p= 0.07, Student's t-test) compared to the thymus expression in the same foetuses (Fig. 5).

Direct tissue damage due to the presence and replication of *N. caninum* at the maternal-foetal interface is a key determinant of foetal mortality related to neosporosis. Notwithstanding, immunological mechanisms in both dams and foetuses also play an essential role in foetal death.

Up-regulation of Th2 (IL-4) and a trend towards up-regulation of Th1 (IFN-γ) was observed in spleen and lymph nodes draining the placenta (UTLN) in infected dams with live foetuses compared to uninfected control dams, reinforcing previous studies (e.g. Almeria et al., 2003;~~,~~ Cantón et al., 2014; Regidor-Cerrillo et al., 2014; Rosbottom et al., 2007). The timing of a Th1 response during pregnancy seems critical for foetal survival and Th2 cytokines may play a significant role in preventing foetal loss caused by unsuitable inflammatory cytokine expression (Rosbottom et al. 2007). In the present study the up-regulated expression levels of IFN-γ and IL-4 in infected dams with live foetuses in spleen and UTLN were consistent with increased production of both

cytokines in cell cultures stimulated with *N. caninum* antigen compared to uninfected dams analyzed in the same animals (Darwich et al., 2016), indicating that changes in mRNA expression correlated with levels of protein production.

A mixed cytokine pattern was also observed in the placenta, with infected dams with live foetuses showing up-regulated expression of Th1, Th2 and Treg cytokines compared to uninfected dams (controls). Infected dams with live foetuses had significantly up-regulated IFN-γ in both the caruncle and cotyledon, up-regulated IL-10 in cotyledon and a trend to up-regulated IL-4 in caruncle. Hence, it seems a threshold IFN-γ response is needed to be beneficial against *Neospora*-infection, as previously suggested (Almería et al., 2014).

Interestingly, while increased expression of Th1 and Th2 was still occurring in response to the infection in infected dams with live foetuses 6 weeks after infection, significant down-regulation of both IL-4 and IL-10 was observed in infected dams with aborted/non-viable foetuses compared to the expression levels in infected dams with live foetuses in spleen and UTLN. No significant differences were observed in cytokine levels between dams with aborted foetuses and those in non-infected control dams. These data could reflect the immunological recovery of cytokine expression levels in these organs after abortion had occurred. Two dams aborted at 14 and 21 days post infection and a third dam presented a mummified foetus upon euthanasia, and this foetus was estimated to have been dead at 28 days post infection (Almeria et al., 2016).

In the infected foetuses, the up-regulated IFN-γ and IL-10 expression observed could be indication of active response against an ongoing infection. The expression of any cytokine in the thymus was significantly lower compared to the levels observed in foetal spleen, a possible indication of delayed development of the immune response of the

thymus in the infection at this stage of gestation. Infected foetuses even have significantly downregulated expression of IL-4 in the thymus compared to control uninfected foetuses. In cell cultures no cytokine production (IFN-γ, IL4) was observed in the thymus in agreement with these results (Darwich et al., 2016).

In summary, *N. caninum* infection of dams at mid-gestation was accompanied by up-regulated expression of Th1, Th2 and Treg at internal immune organs and at placenta level in dams with live foetuses and in their foetuses. On the other hand, downregulation of Th2 and Treg expression levels were observed in internal immune organs in dams with aborted-non viable foetuses compared to those with live foetuses and in similar levels to control dams which suggests an immunological recovery of cytokine gene expression levels in dams a few weeks after an abortion occurred.

**Acknowledgements**

The authors thank Ana Burton for assistance with the English translation. This study was supported by a grant from the Spanish MINECO (AGL2012-39830-C02-01/02) and FEDER. The authors would like to thank the farmers who provided the experimental animals.

References

Almería, S., López-Gatius, F., 2013. Bovine neosporosis: clinical and practical aspects. Res.Vet. Sci. 95, 303-309.

Almería, S., De Marez, T., Dawson, H., Araujo, R., Dubey, J.P., Gasbarre, L.C. 2003. Cytokine gene expression in dams and foetuses after experimental *Neospora*

*caninum* infection of heifers at 110 days of gestation. Parasite Immunol. 25, 383-392.

Almería, S., Araujo, R., Tuo, W., López-Gatius, F., Dubey, J.P., Gasbarre, L.C., 2010. Foetal death in cows experimentally infected with *Neospora caninum* at 110 days of gestation. Vet. Parasitol. 169, 304–311.

Almería, S., Serrano, B., Yàniz, J.L., Darwich, L., López-Gatius, F., 2012. Cytokine gene expression profiles in peripheral blood mononuclear cells from *Neospora caninum* naturally infected dams throughout gestation. Vet. Parasitol. 183, 237-243.

Almería, S., Serrano-Pérez, B., Darwich, L., Araujo, R.N., López-Gatius, F., Dubey, J.P., Gasbarre, L.C. 2014. Maternal and foetal immune response patterns in heifers experimentally infected with *Neospora caninum* in the second trimester of pregnancy-a descriptive study. Vet. Parasitol. 204, 146-152.

Almería, S., Serrano-Perez, B., Darwich, L., Domingo, M., Mur-Novales, R., Regidor-Cerrillo, J., Cabezón, O., Pérez-Maillo, M., López-Helguera, I., Fernández-Aguilar, X., Puig Ribas, M., Ortega-Mora, L.M., García-Ispierto, I., Dubey, J.P., López Gatius, F. 2016. Foetal death in naive heifers inoculated with *Neospora caninum* isolate Nc-Spain7 at 110 days of pregnancy. Exp. Parasitol. 168, 62-69.

Cantón, G.J., Katzer, F., Maley, S.W., Bartley, P.M., Benavides-Silván, J., Palarea-Albaladejo, J., Pang, Y., Smith, S.H., Rocchi, M., Buxton, D., Innes, E.A., Chianini, F. 2014. Cytokine expression in the placenta of pregnant cattle after inoculation with *Neospora caninum*. Vet. Immunol. Immunopathol. 161, 77-89.

Darwich, L. Li, Y., Serrano, B., Mur-Novales, M., Garcia-Ispierto, I., Cabezón, O., López-Gatius, F., Almería, S. 2016. Maternal and foetal cytokine production

in dams naturally and experimentally infected with *Neospora caninum* on gestation Day 110. Res. Vet. Sci. 107, 55-61.

Dubey, J.P., Buxton, D., Wouda, W., 2006. Pathogenesis of bovine neosporosis. J. Comp. Pathol. 134, 267–289.

Leutenegger, C.M., Alluwaimi, A.M., Smith, W.L., Perani, L., Cullor, J.S. 2000. Quantitation of bovine cytokine mRNA in milk cells of healthy cattle by real-time TaqMan polymerase chain reaction. Vet. Immunol. Immunopathol. 77, 275-287.

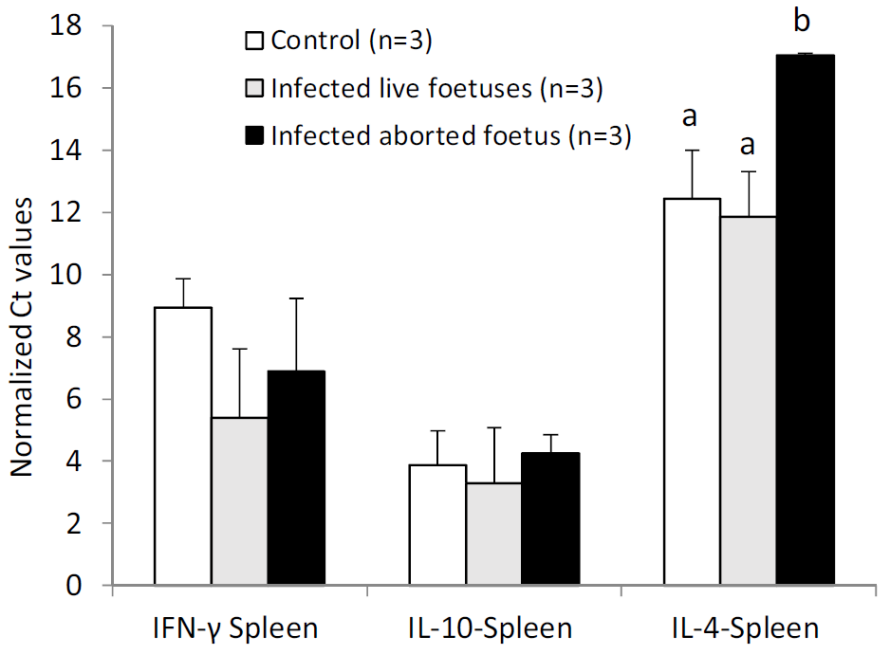
Regidor-Cerrillo, J., Arranz-Solís, D., Benavides, J., Gómez-Bautista, M., Castro-Hermida, J.A., Mezo, M., Pérez, V., Ortega-Mora, L.M., González-Warleta, M., 2014. *Neospora caninum* infection during early pregnancy in cattle: how the isolate influences infection dynamics, clinical outcome and peripheral and local immune responses. Vet. Res. 45, 10.

Rosbottom, A., Guy, C.S., Gibney, E.H., Smith, R.F., Valarcher, J.F., Taylor, G., Williams, D.J., 2007. Peripheral immune responses in pregnant cattle following *Neospora caninum* infection. Parasite Immunol. 29, 219-228.

**Figure legends**

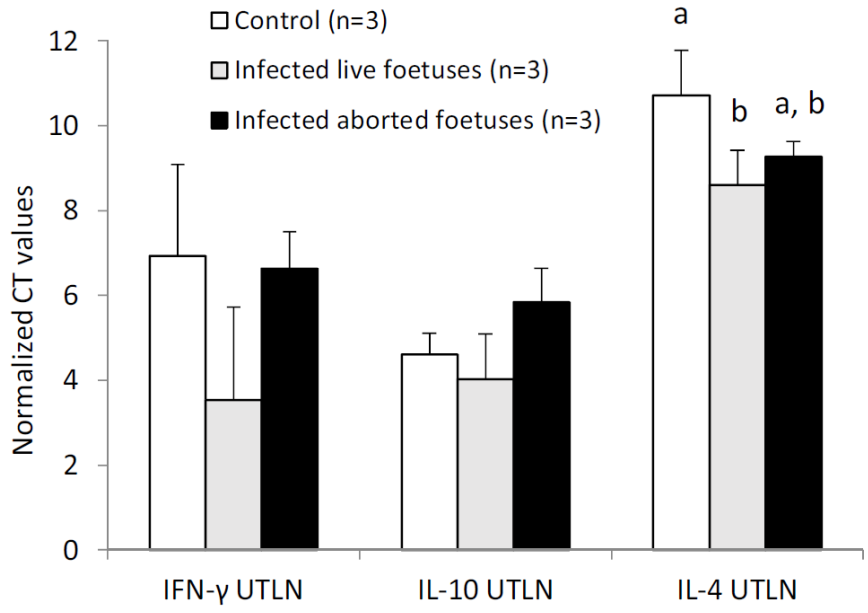
Figure 1. Cytokine gene expression (normalized CT values) among groups (three dams per group) (uninfected dams (controls); *Neospora caninum* experimentally infected dams with live foetuses and experimentally infected dams with dead/aborted foetuses) in the Spleen (A); and in the lymph nodes draining the placenta, Uterine Lymph Nodes (UTLN) (B). The higher the normalized CT value the lower the expression level. Statistically significant differences when different letters among groups.

A


Control (n=3)
Infected live foetuses (n=3)
Infected aborted foetus (n=3)
Normalized Ct values
0
2
4
6
8
10
12
14
16
18
a
a
b
IFN-γ Spleen
IL-10-Spleen
IL-4-Spleen

1

B


Control (n=3)
Infected live foetuses (n=3)
Infected aborted foetuses (n=3)
Normalized CT values
0
2
4
6
8
10
12
a
b
a, b
IFN-γ UTLN
IL-10 UTLN
IL-4 UTLN

2

3

Figure 2. Cytokine gene expression (normalized CT values) showing significant differences among groups (three foetuses per group) (*N. caninum* experimentally infected live foetuses versus control uninfected foetuses) in maternal placenta (caruncle) and in foetal placenta (cotyledon). The higher the normalized CT value the lower the expression level. Statistically significant differences when different letters among groups.

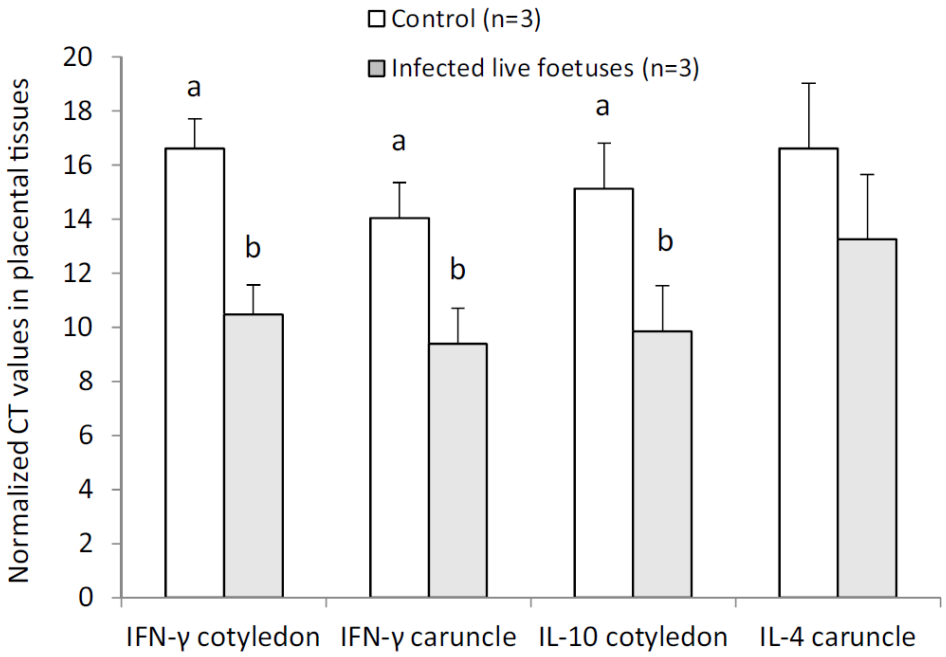


Figure 3. Individual normalized CT values in uninfected control dams (n=3), *Neospora caninum* infected dams with live foetuses (n=3) and in dam #649 which had a dead foetus at euthanasia in Caruncle (maternal placenta). The higher the normalized CT value the lower the expression level.

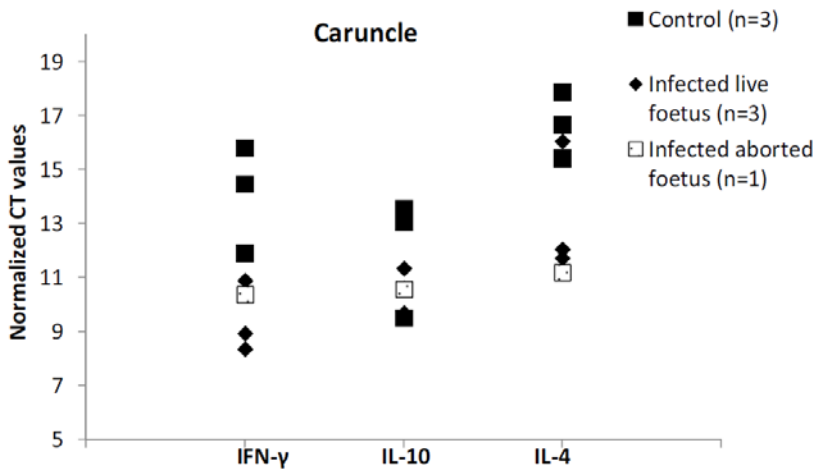


Figure 4. Cytokine gene expression (normalized CT values) showing significantly differences in expression levels among groups (three animals per group) (*N. caninum* experimentally infected live foetuses versus control uninfected foetuses) in spleen and thymus. The higher the normalized CT value the lower the expression level. Statistically significant differences when different letters among groups.

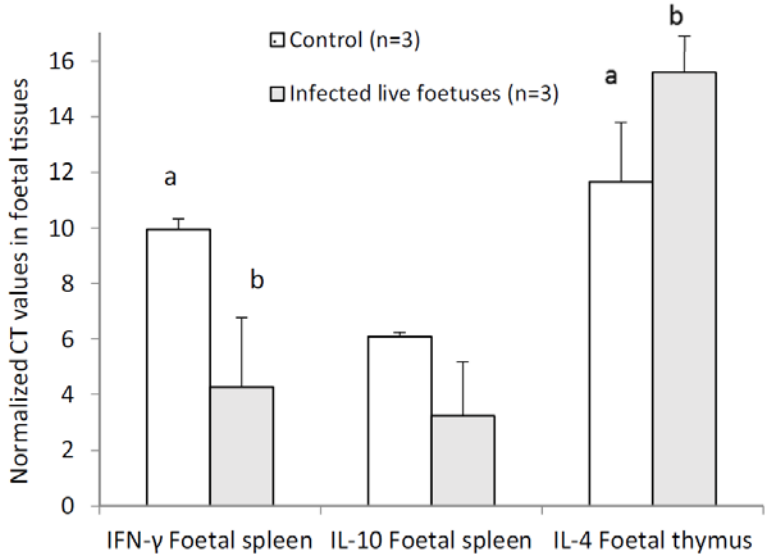


Figure 5. Comparison of cytokine expression levels (normalized Ct values for IFN, IL-10 and IL-4) in Thymus and Spleen in foetuses analyzed in the same assay. The higher the normalized CT value the lower the expression level. Statistically significant differences when different letters among groups.